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Odorant Inhibition of the Olfactory Cyclic Nucleotide-gated Channel with a Native Molecular Assembly

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Human olfaction comprises the opposing actions of excitation and inhibition triggered by odorant molecules. In olfactory receptor neurons, odorant molecules not only trigger a G-protein–coupled signaling cascade but also generate various mechanisms to fine tune the odorant-induced current, including a low-selective odorant inhibition of the olfactory signal. This wide-range olfactory inhibition has been suggested to be at the level of ion channels, but definitive evidence is not available. Here, we report that the cyclic nucleotide-gated (CNG) cation channel, which is a key element that converts odorant stimuli into electrical signals, is inhibited by structurally unrelated odorants, consistent with the expression of wide-range olfactory inhibition. Interestingly, the inhibitory effect was small in the homo-oligomeric CNG channel composed only of the principal channel subunit, CNGA2, but became larger in channels consisting of multiple types of subunits. However, even in the channel containing all native subunits, the potency of the suppression on the cloned CNG channel appeared to be smaller than that previously shown in native olfactory neurons. Nonetheless, our results further showed that odorant suppressions are small in native neurons if the subsequent molecular steps mediated by Ca^{2+} are removed. Thus, the present work also suggests that CNG channels switch on and off the olfactory signaling pathway, and that the on and off signals may both be amplified by the subsequent olfactory signaling steps.

The Journal of General Physiology

INTRODUCTION

Olfactory signal transduction begins with the binding of odorant to the receptor, which triggers the activity of a G-protein, and then stimulates the adenylate cyclase to make cAMP. The intracellular cAMP then opens the olfactory CNG channel, which depolarizes the neuron and allows the influx of Ca^{2+} into the cell (Kurahashi and Yau, 1994; Schild and Restrepo, 1998; Firestein, 2001). The increase of intracellular Ca^{2+} results in an activation of the Ca^{2+}-activated Cl^- current, which amplifies the signal and further depolarizes the olfactory receptor neuron (Kurahashi and Yau, 1993; Lowe and Gold, 1993). For olfactory sensations, odorant is not only a stimulator but also a suppressor (Matthews and Reisert, 2003). The suppression of the olfactory signal by odorant molecules was first revealed by a "double-puff" experiment (Kurahashi et al., 1994). In such an experiment, the first puff of the odorant induced an inward current, but if the odorant was applied at the peak of the current, a strong current suppression by the second puff of the odorant was observed. It was suggested that this suppression comes from a direct inhibition of CNG channels by odorant molecules, because there was almost no delay in the onset of the current suppression upon the application of the second puff of the odorant (Kurahashi et al., 1994).

Although attempts to test a direct odorant inhibition on olfactory CNG channels have been performed, the experiments were performed in native neurons that contain all the signaling molecules of the olfactory transduction pathway (Yamada and Nakatani, 2001). The suggestions that Ca^{2+}-activated K^+ channels may mediate an odorant-induced inhibitory response (Delgado et al., 2003), and that some odorants can act as antagonists of odorant receptors (Oka et al., 2004) complicate the issue. Since applying odorant molecules to the native neuron inevitably influences the activity of all signaling molecules, it is difficult to unambiguously demonstrate the odorant inhibition on the CNG channel. In the present study, we examine the olfactory CNG channels in a heterologous expressing system and show that odorants indeed inhibit the olfactory CNG channel. The homo-oligomeric channel entirely formed by the principal subunit (CNGA2) is less sensitive to odorant inhibition than the hetero-oligomeric channels formed by coexpressing CNGA2 with CNGA4, CNGB1, or both. Our results also show that the inhibition on the cloned channel appears to be weaker than the current suppression in native olfactory neurons, suggesting that the inhibition on the CNG channels may also be amplified by subsequent signaling steps.

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Abbreviations used in this paper: AA, amyl acetate; ANI, anisole; CIN, cineole; CNG, cyclic nucleotide-gated; IAA, isoamyl acetate; LIM, limonene.

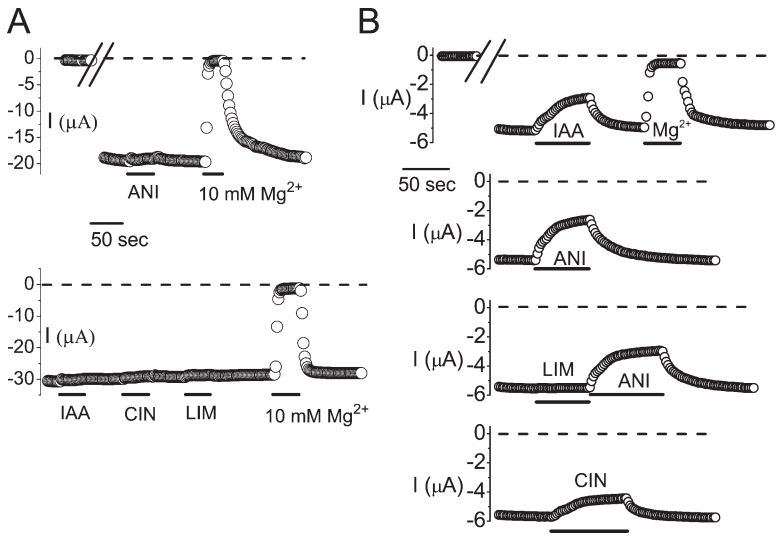

Figure 1. Comparing the odorant inhibition in the homo-oligomeric and hetero-oligomeric CNG channels. (A) Sensitivity of the homo-oligomeric channel formed by subunit A2 to odorants at a concentration of 0.5 μl/ml. Top panel tests ANI suppressions. The gap in the recording represents the 30-min incubation of the oocyte in the solution with 100 μM 8Br-cGMP. Bottom panel, experiments using IAA, CIN and LIM in another oocyte. Three other oocytes showed similar results. (B) Sensitivity of the hetero-oligomeric channel to odorants. All recordings were from the same oocyte. The channels were generated by injecting RNAs of subunits A2, A4, and B1 (RNA ratio, 2:1:1) into the oocyte. IAA, ANI, LIM, and CIN were all at a concentration of 0.5 μl/ml. Mg^{2+} was 10 mM. Recording gap on top represents the 8Br-cGMP incubation period. Three other oocytes showed similar results.

MATERIALS AND METHODS

Molecular Biology and Channel Expression

To isolate olfactory CNG channels from other olfactory signaling molecules, we expressed channels in *Xenopus* oocytes. The procedures in harvesting and injecting oocytes were published previously (Chen, 1998). The cDNAs of the rat olfactory CNG channel subunits, CNGA2, CNGA4, and CNGB1, all subcloned in the pGEMHE vector, were gifts from B. Zagotta and S. Gordon (University of Washington, Seattle, WA). RNAs were made from these cDNAs using T7 mMessage mMachine kit (Ambion). Four combinatorial ways of injecting RNAs were employed: subunit CNGA2 alone (A2); subunit CNGA2 and CNGA4 (A2 + A4); subunit CNGA2 and CNGB1 (A2 + B1); and subunit CNGA2, CNGA4, and CNGB1 (A2 + A4 + B1). For RNA mixing, the ratio of RNAs of A2:A4:B1 were 2:1:1 (Zheng and Zagotta, 2004). Normally, recordings were performed 2–5 d after the RNA injection.

Electrophysiological Recordings of Cloned CNG Channels

Whole oocyte current was recorded by standard two-electrode voltage clamp techniques using an oocyte clamp amplifier (725C, Warner Instruments, Inc.). The current was digitized online into the computer using Digidata 1200 A/D board controlled by pClamp6 software (Axon Instruments/Molecular Devices). The recording electrodes had resistance of 0.2–1 MΩ when filled with 3 M KCl. Bath solution (solution A) contained (in mM) 100 NaCl, 0.5 EDTA, 1 niflumic acid (to suppress the endogenous Ca^{2+}-activated Cl^- channels on the oocyte membrane), and 5 HEPES, pH 7.4. To activate the channels from the intracellular side, we soaked oocytes in 100 μM membrane-permeable 8Br-cGMP in solution A for at least 30 min. To monitor the oocyte current, the membrane potential was held at −10 or 0 mV, and a voltage pulse to +60 mV followed by a pulse to −60 or −80 mV was given every 2–6 s. After 8Br-cGMP concentration was built up within the oocyte, the intracellular 8Br-cGMP concentration was in a presumably saturated concentration because even after removing extracellular 8Br-cGMP for tens of minutes, the current was still very stable. The current was inhibited by extracellular Mg^{2+}, with a dose-dependent inhibition curve showing a half-blocking concentration of 45.1 ± 4.7 μM ($n = 3$, unpublished data). This value is similar to that reported previously (Heginbotham et al., 1992; Root and MacKinnon, 1993), indicating that this 8Br-cGMP–induced, Mg^{2+}-blockable current indeed comes from CNG channels.

Odorant molecules included isoamyl acetate (IAA), anisole (ANI), cineole (CIN), and limonene (LIM) (Sigma-Aldrich), which were directly mixed with the recording solutions upon use. Judging from the rate of Mg^{2+} inhibition of the current, the solution exchange was completed within 5–8 s. Each application of the odorant solution was at least 1 min, and the current amplitude was determined when the inhibition reached a steady state (normally after 30 s of the odorant application). To subtract the leak current, we applied 10 mM $MgCl_2$ in each experiment. The percentage of odorant inhibition was calculated by normalizing the amount of odorant-inhibited current to the current blocked by 10 mM Mg^{2+}. Mean values and SEM were used to present the results.

Recordings of Native Olfactory Neurons

Olfactory receptor neurons were dissociated from collagenase-treated newt olfactory epithelia, after the procedure described previously (Takeuchi and Kurahashi, 2003; Takeuchi et al., 2003). The superfusate contained (in mM) 110 NaCl, 3.7 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 15 glucose, 1 pyruvate, pH 7.4. Membrane currents were recorded in whole-cell mode (Hamill et al., 1981) with Axopatch 1D or 200B amplifiers (Axon Instruments, Inc.), low-pass filtered at 0.5 kHz, digitized by an A/D converter (sampling frequency, 1 kHz), and finally saved on to a computer (PC9821; NEC or IBM compatible PC). The patch pipette was made from borosilicate glass capillaries (World Precision Instruments) and filled with a Cs-solution containing (in mM) 119 CsCl, 1 $CaCl_2$, 5 EGTA,10 HEPES (pH 7.4). The pipette resistance was 10–15 MΩ.

The odorants amyl acetate (AA) and CIN were from Katayama Co., and lilial (LIL) was from Takasago Co. They were dissolved in DMSO and then diluted in the superfusate at 1 mM concentration with final DMSO concentration 0.2%. The odorant solution was applied to the cilia of olfactory neurons from a puffer pipette (tip diameter ~1 μm). The pressure was manipulated to change the dose of the stimulants (Ito et al., 1995).

Solitary olfactory neurons were loaded with 1 mM caged cAMP (adenosine 3′,5′-cyclic monophosphate, P1-(2-nitrophenyl)ethyl ester; Calbiochem) diffused from the whole-cell pipette as previously described (Takeuchi and Kurahashi, 2005). A UV component with a wide wavelength (light source: 100 W xenon lamp) was focused with an epifluorescent system onto the cilia of the neurons. Light on and off were regulated by the magnetic shutter. The light intensity was specified by a wedge filter and was indicated as relative values in reference to the maximum intensities being 1.0.

RESULTS

The olfactory CNG channel is a tetramer including three different types of subunits. CNGA2 is the principal subunit (Dhallan et al., 1990), and this subunit alone can form functional channels, although such a homo-oligomeric channel is different from the native channel in various functional properties. Two other subunits also participate in forming the native olfactory CNG channel. One of them, now called CNGA4, was found to significantly increase the apparent cAMP affinity for the channel (Bradley et al., 1994; Liman and Buck, 1994). The other auxiliary subunit, CNGB1, was originally found in the rod photoreceptor (Chen et al., 1993) and was later shown to also be present in the olfactory receptor neuron (Bonigk et al., 1999). These two auxiliary subunits cannot form functional channels by themselves, but when coexpressed with the principal subunit, they alter the functional properties of the channel (Bradley et al., 1994; Liman and Buck, 1994; Bonigk et al., 1999; Bradley et al., 2001).

Using channel expressions in oocytes, we first tested sensitivity of CNGA2 homo-oligomeric channels to odorants ANI, IAA, CIN, and LIM, all at a concentration of 0.5 μl/ml (Fig. 1 A). Unexpectedly, none of the odorants had detectable effects on the 8Br-cGMP–induced current, although the odorant concentration was equal to, or higher than, that used in the previous experiment on the olfactory neuron (Kurahashi et al., 1994). To mimic the native olfactory CNG channel more closely, we expressed channels by coinjecting the RNAs of these three subunits into oocytes (Fig. 1 B). In contrast to the homo-oligomeric channel, three of the four tested odorants inhibited the hetero-oligomeric channel at the same concentration. Only the odorant LIM had no effect. This is consistent with the early study in the native olfactory neuron (Kurahashi et al., 1994). Thus, in addition to several previous functional roles identified for the accessory subunits (Bradley et al., 1994; Liman and Buck, 1994; Bonigk et al., 1999; Bradley et al., 2001), subunit A4 and B1 also help increase the sensitivity of the olfactory CNG channel to odorant inhibition.

To quantify the odorant suppression, we tested the sensitivities of the channels to different concentrations of odorant (Fig. 2). With odorant concentrations <2 μl/ml, the inhibition course was relatively normal, the current was suppressed upon odorant application and recovered nearly to the control level after the odorant was washed out. The suppression was dose dependent for ANI (Fig. 2 A), IAA, and CIN (recording traces not shown, but see below). When higher concentrations (2 or 5 μl/ml) of the odorant were applied, the current inhibition was more prominent, but the oocyte sometimes also became leaky (see arrow at the end of the recording in Fig. 2 A). The leakiness of the membrane

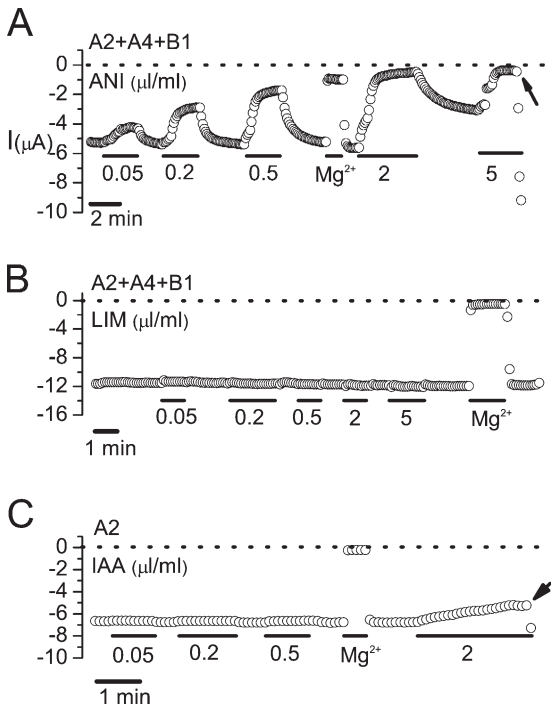

Figure 2. Dose dependence of the odorant inhibition of olfactory CNG channels. (A) Suppression of the olfactory CNG channel (A2 + A4 + B1) by various concentrations of ANI. The labeling of "Mg^{2+}" indicates the application of 10 mM $MgCl_2$. Arrow at the end of the recording indicates a membrane breakdown, which is followed by a sudden increase in leak current. (B) Low sensitivity of the triple-subunit channel to LIM. Note that even at the highest concentration, 5 μl/ml, LIM does not have an appreciable suppression on the channel. The oocyte is also intact at such a high concentration of LIM. (C) Suppression of the homo-oligomeric channel (CNGA2) by IAA. The current is not inhibited by IAA at concentrations ≤0.5 μl/ml. At 2 μl/ml, a slight and slow suppression can be detected although the oocyte becomes leaky at this concentration of IAA.

occurred in a similar range of concentrations for all three odorants that inhibited the CNG channels. At 2 μl/ml, 23 out of 68 (33.8%) recorded oocytes became leaky, while >70% (18 out of 23) tested oocytes were leaky at 5 μl/ml of odorants. On the other hand, LIM had a negligible effect in inhibiting channels with all combinatorial subunits, and no oocytes treated with LIM became leaky even at the highest concentration of 5 μl/ml (Fig. 2 B). When the concentration of ANI, IAA, or CIN was 2–5 μl/ml, the current mediated by the homo-oligomeric channel composed of only subunit A2 was also affected (Fig. 2 C).

To compare the sensitivity of the homo-oligomeric and hetero-oligomeric channels to various odorants, we systematically tested the olfactory CNG channels with four combinatorial subunit expressions: subunit A2 only, subunit A2 + A4, subunit A2 + B1, and subunit A2 + A4 + B1 (Fig. 3). We quantified the inhibition only up to the odorant concentration of 2 μl/ml, because the majority of the oocytes became leaky at 5 μl/ml.

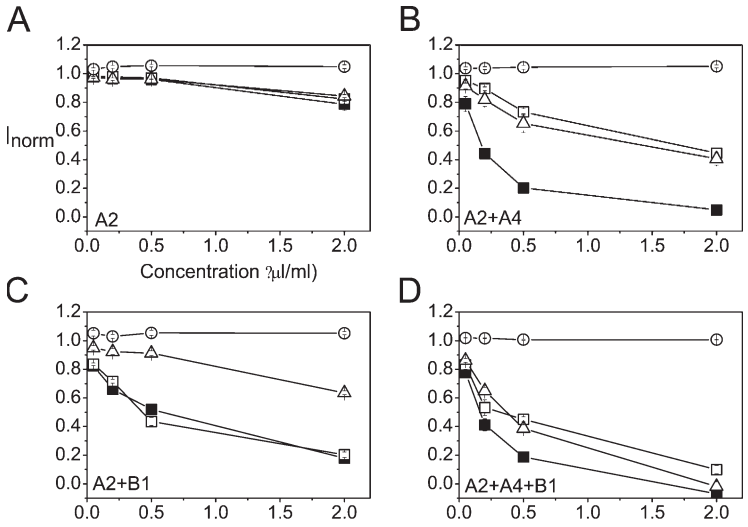

Figure 3. Dose–suppression curves of various types of olfactory CNG channels. (A) Homo-oligomeric CNG channels formed entirely by subunit CNGA2. The number of oocytes (n) tested are 4, 3, 4, and 5 for ANI, IAA, CIN, and LIM, respectively. (B) Hetero-oligomeric channels formed by subunits A2 and A4 (A2 + A4); n = 3–5. (C) Hetero-oligomeric channels (A2 + B1); n = 4–5. (D) Hetero-oligomeric channels (A2 + A4 + B1); n = 3–4. In all four panels, symbols are as follows: solid squares, ANI; open squares, IAA; open triangles, CIN; open circles, LIM. Data represented as mean ± SEM.

It can be seen that LIM has no effect throughout various concentrations in all four types of channels. The inhibition by ANI, IAA, and CIN is dose dependent, but the effect is much weaker in the homo-oligomeric channel; only at the concentration of 2 μl/ml can an appreciable inhibition be detected. In the other three types of channels, the inhibition can be detected as low as 0.05 μl/ml. At the concentration of 0.5 μl/ml, the current is inhibited by 48–81%, 27–57%, and 8.9–61% for ANI, IAA, and CIN, respectively (see Fig. 3, B–D, for the percentage inhibition by each odorant in different types of channels). There may be a slight difference among the three hetero-oligomeric channels in terms of the inhibition potency. The channel containing all three subunits appears to have the highest sensitivity, although the difference is only subtle.

Even in the channel with the highest sensitivity to odorants, i.e., the hetero-oligomeric channel containing all three subunits, the potency of odorant inhibition appears to be lower than that shown in the olfactory neuron (Kurahashi et al., 1994). For example, at the concentration 0.2 μl/ml, cloned channels were suppressed by ~30%, while in the olfactory neuron only a brief puff of 0.2 μl/ml AA suppressed the odorant-induced current by ~60% (Kurahashi et al., 1994). To further address such a difference, we studied olfactory neurons from newts using whole-cell patch configurations (Fig. 4). The membrane potential of the olfactory neuron was clamped at −50 mV (Fig. 4 A, top panel) or +120 mV (where little Ca influx is expected, Fig. 4 A, bottom panel), and the odorant LIL at 0.2 μl/ml was puff-applied on the cell during the period when the current was induced by uncaging cAMP intracellularly to activate the native CNG conductance. The inhibition at −50 mV was fast and large, while the inhibition of the same neuron at +120 mV was small, even with a longer puff. The collective results in Fig. 4 B confirm that the odorant inhibition of the odorant-induced current is more potent at −50 mV than at +120 mV. The difference was not due to the voltage-dependent channel blockade because suppression was observed for the same extent at both +60 and −60 mV when examined with cloned channels (unpublished data). It is likely that the difference may result from the diminished Ca^{2+} influx at +120 mV.

DISCUSSION

We have used the *Xenopus* oocyte expression system to examine if odorant molecules can inhibit the olfactory CNG channels. The observed current is mostly contributed by the olfactory channel, because it appears only after the oocyte is soaked in a membrane-permeable cyclic-nucleotide analogue, 8Br-cGMP. We have also used the Mg^{2+} blockade to confirm the current. Our experiments therefore provide a simple system in which only the olfactory CNG channel exists, but not other components in the olfactory signaling pathway. Under such a condition, we have demonstrated that IAA, ANI, and CIN inhibit the channel, while LIM has very little effect. This is consistent with the results from the native olfactory neuron that LIM does not suppress the odorant-induced current (Kurahashi et al., 1994).

For those odorants that inhibit the olfactory CNG channel, the homo-oligomeric channel formed entirely by CNGA2 is less sensitive to the odorant inhibition than the hetero-oligomeric channels in which CNGA2 is coexpressed with CNGA4, CNGB1, or both. This phenomenon is interesting because the auxiliary subunits CNGA4 and CNGB1 are also pivotal for other channel properties, such as increasing Ca^{2+} permeability and speeding up the channel modulation by Ca^{2+}-calmodulin (Bradley et al., 1994; Liman and Buck, 1994; Bonigk et al., 1999; Bradley et al., 2001). The difference in the inhibition potency between the homo- and the hetero-oligomeric channels could have several explanations.

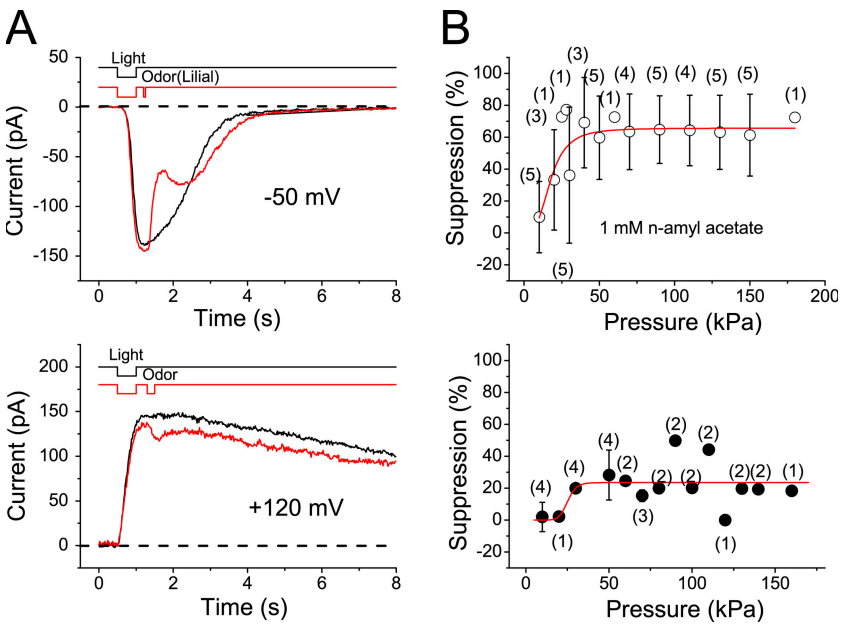

Figure 4. Sensitivity of odorant-induced current in the olfactory neuron clamped at two voltages. Cells that did not respond to the applied odorant were selected for experiments. (A) Suppression of the cAMP-induced current by LIL. Cytoplasmic cAMP was jumped by a photolysis of caged cAMP. The concentration of LIL used to suppress the current was 0.2 μl/ml. Top, V_m = −50 mV; bottom, V_m = +120 mV. The suppression on the basal current was subtracted from the data. (B) Suppression percentage versus the pressure applied to the puff pipette. Odorant, AA (0.1 μl/ml). Top, V_m = −50 mV. Solid curve was Hill fitting of the data points with $K_{1/2}$ = 18 kPa. Bottom, V_m = +120 mV. Hill fitting shows $K_{1/2}$ = 25 kPa. Error bars represent SD, and numbers in parentheses indicate the numbers of cells tested.

First, the odorant binding sites for the channel inhibition may reside in CNGA4 and CNGB1, but not in CNGA2 subunit. Alternatively, perhaps the odorant-binding sites reside in between two subunits, and only the sites contributed by the accessory subunit are able to accommodate the odorant molecules. However, these two possibilities, which evoke specific binding sites on the channel protein, are less compelling because IAA, CIN, and ANI do not share a common molecular structure. The lack of specificity may instead suggest an effect on the lipid membrane because odorants are quite hydrophobic. Alterations of the lipid raft, or the changes in the hydrophobic mismatch between lipids and proteins, have functional consequences on membrane proteins and ion channels (Lundbaek and Andersen, 1999; Hwang et al., 2003). For hetero-oligomeric CNG channels, the asymmetrical arrangement of the four subunits may render the gating of the channels easier to be altered by the distorted lipid bilayer. The suggestion of the lipid effect comes from at least two lines of evidence. First, the effective concentration to inhibit the hetero-oligomeric channel is already very high (0.5 μl/ml ≈ 5–10 mM for these odorants), within an order of magnitude to the concentration that breaks down the oocyte membrane (see Fig. 2). It is therefore conceivable that the low, but effective, concentrations of odorants may have already altered the lipid membrane structure. The observation that LIM, which does not inhibit CNG channels, causes no membrane breakdown appears to further support this argument. The other line of the evidence for the lipid effect comes from the literature. Odorant molecules have been shown to inhibit not only the olfactory CNG channels, but also the CNG channels in photoreceptors (Kawai and Miyachi, 2000), the voltage-gated ion channels in olfactory receptor neurons (Kawai et al., 1997), and even the gramicidin channel in artificial lipid bilayers (Andersen et al., 1999), all at comparable concentrations (hundreds of micromolar to low millimolar). The lipid components in these experiments may not be exactly the same, but the inhibition on these channels could all be mediated through the perturbation of lipid membranes.

Although the present study clearly demonstrates that odorants indeed inhibit current through CNG channels, the inhibition in the cloned channels appears not as potent as that observed in the native neurons. The odorant-induced current in the olfactory neuron is known to have at least two components. One is contributed by CNG channels, and the other comes from the Ca^{2+}-activated Cl^- channel (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993; Matthews and Reisert, 2003). It is the Ca^{2+} ions through CNG channels that open Ca^{2+}-activated Cl^- channels and nonlinearly amplify the olfactory signal. Therefore, the apparently more potent odorant inhibition observed in olfactory neurons could result from the disappearance of the Ca^{2+}-activated Cl^- current due to the reduced Ca^{2+} influx resulting from the closure of CNG channels. A direct inhibition on the Ca^{2+}-activated Cl^- channel is also possible because perturbation of the cell membrane by odorants could potentially affect other ion channels. A proof of the latter possibility may require the heterologous expression approach as demonstrated in this study. Nonetheless, both possibilities are consistent with the observation of small odorant suppression on the native neuron when the Ca^{2+} influx is stopped by a very positive membrane potential (Fig. 4). Although suppression mechanisms from other signaling molecules cannot be ruled out, it is likely that the inhibition of the CNG channel and the subsequent amplified olfactory

signal contributes to a significant portion of the odorant suppression.

The effective odorant concentrations in inhibiting the olfactory signals in native olfactory neurons (Fig. 4) appear to be higher than those expected from human olfactory experience, raising a concern if this inhibition is of any physiological significance. Our experiments, however, showed roughly equal concentration ranges effective for odorant excitation and suppression in native olfactory neurons. For example, under the same condition, the least effective concentration ranged between 10 and 20 kPa for suppression (Fig. 4 B), and between 8 and 30 kPa for excitation (not depicted), when tested with CIN, AA, LIL (duration, 100 ms; all 1 mM, equal to 0.1–0.2 μl/ml). It was suggested that odorant molecules can be enriched in the olfactory epithelium through binding to the odorant-binding proteins (Pevsner et al., 1986; Tsuchihara et al., 2005). If exposing intact olfactory epithelia to air-borne odorant can generate enough odorant molecules in the olfactory epithelia to activate the neuron, it is conceivable that the same enriched odorant concentration may mediate the inhibition on CNG channels. Physiologically, however, it remains to be explored if such an odorant-enriched mechanism in the native tissue produces a homogeneous or a local increase of the odorant concentration that perturbs the membrane of the olfactory receptor neurons.

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